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A NEW COLOR MEDIUM FOR THE ISOLATION AND DIFFERENTIATION OF STREPTOCOCCI.*

D. D. TODD.

(*From the Memorial Institute for Infectious Diseases, Chicago.*)

It is the purpose of this paper to give briefly the results of tests of the action of various strains of streptococci on various dyes in sugar media. The work was commenced on the assumption that some definite color reaction might be found characteristic of different strains of the cocci depending on the action of the products of fermentation on the dyes.

Previously but little has been said on this basis of differentiation of streptococci, altho the principle involved is a common one, as seen in the various color media for the isolation of B. typhosus and other bacteria. A plate method of great importance is that of Schottmüller[1] in which, by the use of blood-agar, he differentiated Strept. pyogenes, Strept. viridans, and Strept. mucosus. This method, in reality, represents two properties of the cocci, the hemolytic power of Strept. pyogenes and the fermentative power of Strept. viridans, for Ruediger[2] has shown that the green coloration of the latter is probably due to the action of the coccus on the muscle sugar in the agar.

Gordon[3] experimented with a watery solution of neutral red which he added to broth as a means of differentiation. He found, in anaerobic cultures, that Strept. brevis (Lingelsheim) reduced the dye with the production of a green fluorescence, while Strept. longus (Lingelsheim) and Strept. pyogenes reacted negatively. Fregonneau[4] tested methyl orange and found no characteristic color reaction for numerous bacteria, including the streptococcus.

In carrying out my tests, about an equal number of strains of Strept. pyogenes and Strept. viridans (Schottmüller) were isolated from the

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[1] *Münch. med. Wchnschr.*, 1903, 50, p. 909.

[2] *Jour. Infect. Dis.*, 1906, 3, p. 663.

[3] *Centralbl. f. Bakt.*, Orig., 1904, 35, p. 271.

[4] *Ibid.*, Orig., 1909, 49, p. 276.

mouth by the use of the blood-agar plate method.[1] Each strain was inoculated in a double set of media, and only those were selected which corresponded in growth in both sets. A working classification was made by taking the degree of fermentative action in litmus milk (see Table 1). This gave four groups, and the numerical frequency with which the other cultural characteristics agreed placed the strains in different sub-groups. A number of the strains of each sub-group were tested on the dye sugar media. No attempt was made to apply Gordon's tests[2] or to arrange the cocci according to the grouping of Andrewes and Horder,[3] it being the desire to make the classification of the cocci, based on their fermentative powers, as simple as possible. This was necessary, if a plate method of differentiation was to be found in which only one sugar could be used and that necessarily being one commonly fermented by all strains of streptococci.

TABLE 1.
Classification of Streptococci.

Milk Culture	Number of Strains	Gram Positive	Morphology			Colonies in Blood-Agar Plates		Broth Culture			Gelatin	
			Elongated		Round			Turbid	Clear			
			Long Chains	Short Chains	In Chains	Hemolysis	Green	Uniform	Diffusible	Flocculent	Growth	No Growth
No coagulation												
Slight acidity	12	12	4	1	7	10	2		11	1	4	8
Marked acidity	23	23	8	11	4	19	4	3	14	6	12	11
Coagulation												
Deep red	26	26	7	14	5	12	13	10	7	7	19	7
Complete decolorization	13	13	1	11	1	11	2	4	6	3	11	2

The media were made as usual. A large amount of sugar-free agar was prepared so as to have a uniform medium on which to make the tests. To this two per cent sugar was added. The sugars were dextrose, lactose, and saccharose, which are fermented by all strains of streptococci. Later it was found that any one of the sugars gave the same reaction with dyes and accordingly lactose was used exclusively.

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[1] The strains of *Strept. viridans* were grown on inulin agar, in addition to the morphological and cultural tests, to differentiate them from the pneumococcus according to the method of Ruediger, *Jour. Infect. Dis.*, 1906, 3, p. 183.

[2] *Lancet*, 1905, 2, p. 1400.

[3] *Ibid.*, 1906, 2, pp. 708, 775, 852.

As to the amount of dye added, considerable experimentation was required. Rothberger[1] reported the use of a large number of dyes in liquid media in an attempt to differentiate various bacteria. The amount of dye he added was just a sufficient number of drops of saturated watery solution of the dye to color the broth. This idea was used as a guide to the first determination of the approximate amount of dye to add to the sugar medium. A one per cent solution of the dyes was accurately made and measured amounts of this were added to a definite volume of liquid agar to just give a trace of the characteristic color of the dye to the medium. Several strains of the streptococci were then plated out in each dye medium, and the amount of dye per liter of agar, in which a good growth of streptococci was obtained, is shown in Table 2. The medium was sterilized for three successive days in the Arnold sterilizer. Sterile beef serum to the amount of 2 c.c. was added to each tube of media (7–8 c.c.) just before plating. This was found to make the growth of the cocci more rapid, and to add to the characteristic appearance of the colonies by the formation of a milky-like haze around the colonies, which is probably due, as explained by v. Hecht and Hulles,[2] to the formation of an albuminous precipitate as the result of the acid formation from the action of the cocci on the sugar.

TABLE 2.
DYES (GRÜBLER) TESTED IN AGAR MEDIUM.

Acid Stains	c.c. per Liter*	Basic Stains	c.c. per Liter*
Congorot	5.0	Bismarck braun	10.0
Corallin	3.0	Chrysoidin	3.0
Eosin	3.0	Dahlia	2.0
Indigcarmin	3.0	Fuchsin	2.0
Indulin	3.0	Janusgrün†	2.0
Metanilgelb	5.0	Jod grün	2.0
Nigroisin	5.0	Magdalaroth	5.0
Rubin S	5.0	Magentaroth	2.0
Säurefuchsin	5.0	Malachitgrün†	2.0
Säureviolett	5.0	Methylengrün†	2.0
Wasserblau	10.0	Methylenblau†	0.2
		Neutralroth	0.4
		Phloxinroth	3.0
		Safranin	3.0
		Toluidinblau	3.0

* One per cent solution of dye. † Pneumococci failed to grow.

After testing various dyes, three were found which gave a definite but a similar color colony with the different strains of streptococci, the dyes being neutral red, rubin S, and acid fuschin. As the three dyes gave similar color colonies, I confined myself to the use of the neutral red and in lactose agar medium.

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[1] *Centralbl. f. Bakt.*, Orig., 25, pp. 15, 72.

[2] *Ztschr. f. Hyg.*, 1909, 63, p. 113.

The different groups of streptococci (Table 1) were repeatedly tested in the neutral red lactose agar medium and only two distinct color colonies were found. On classification of the strains according to these color colonies, the latter were found to represent exactly Strept. pyogenes and Strept. viridans (Schottmüller), or the two types of strains originally isolated. It thus appeared that in the use of the neutral red lactose agar medium lay a method of isolation and differentiation of Strept. pyogenes and Strept. viridans. It further suggested its possible use for the differentiation of allied species.

A number of strains of Strept. mucosus were isolated and tested on the color medium and found to produce a distinct color colony. Five strains of pneumococcus[1] were tested and found to produce color colonies that could be differentiated from the streptococcus.

The deep colonies were not definitely characteristic; the surface colonies appeared as follows:

Strept. pyogenes.—Colonies 1 mm. or less in size, deep red in color with a barely perceptible pink border, margin smooth and sharply defined. After 18–24 hours a slight milky-like haze surrounds the colony, and irregularly spreads from the colony with further incubation.

Strept. viridans (Schottmüller).—Colonies 1–3 mm. in size, red center with a pink border $\frac{1}{5}$–$\frac{1}{3}$ width of center, margin smooth with surrounding large area of milky-like haze. With over 50 colonies the haze or opacity extends entirely over the plate.

Strept. mucosus (Schottmüller).—Colonies 4–5 mm. in size, pink center with white border equal to width of center or greater, margin very irregular and serrated, slight surrounding milky-like haze which increases slowly with incubation.

Pneumococcus.—Colonies 2–3 mm. in size, red center with a narrow white border, margin smooth and sharply defined, slight surrounding milky-like haze which increases but little with incubation.

I have repeatedly isolated the above types of cocci, according to the description of the colonies given, using the neutral red lactose medium to which has been added the beef serum. My work has been confined solely to the bacteria of the mouth, for which, at least, I hold the medium to be of some value for the isolation and differentiation of streptococci and pneumococci.

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[1] Kindly given by Dr. F. C. Rosenow.